JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Development of a Novel Adsorptive Membrane Chromatographic Method for the Fractionation of Polyphenols from Bilberry

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ABSTRACT: A novel membrane chromatographic method with a membrane adsorber (Sartobind S) has been developed on the laboratory scale that allows a fractionation of bilberry (*Vaccinium myrtillus*) constituents into the following three groups of polyphenols: anthocyanins, copigments, and polymers. By using this methodology, a pure anthocyanin fraction free of other copigments and polymeric phenols can be obtained. Using this approach, it provides fractions allowing a more thorough testing of the biological effects of the individual groups of bilberry polyphenols as well as the study of possible synergistic effects between these different groups of bioactive constituents from bilberry.

KEYWORDS: *membrane chromatography, Sartobind S, bilberry, Vaccinium myrtillus, polyphenols, anthocyanins, copigments, polymeric phenols*

INTRODUCTION

Polyphenols are bioactive secondary plant constituents, which are formed by the shikimic acid and acetate pathway.[1,2] They contribute to the color and flavor of fruits and many other plants, thus attracting insects for pollination and seed dispersal.[3] Many polyphenols are formed stress-induced being part of a defense system against pathogenic attack or wounding of the plant by herbivores.[4] Because of the high antioxidant activity,[5−8] polyphenols are known to protect the human body against reactive oxygen species and highly reactive lipid peroxidation products.[9] In this way, they have a protective effect on the development of cardiovascular diseases and cancer.[10−13] By inhibiting the enzyme lipoxygenase, bilberry polyphenols (*Vaccinium myrtillus* L.) show beneficial effects in the treatment of inflammatory diseases such as ulcerative colitis or Crohn's disease.[14] Further bioactivity studies were directed at the possible suppression of colon cancer[15−18] and improvement of night vision.[19,20] The polyphenolic fraction of bilberry consists of anthocyanins, copigments like phenolic acids and flavonoids (colorless phenolic compounds, which can afford a color contribution by copigmentation of anthocyanins), and polymeric phenolic compounds.[21−23] As a prerequisite for evaluating the biological activity of these individual groups of polyphenols, and possible synergistic effects between them, efficient preparative separation methods are required. The yet available preparative separation procedures only allow a partial enrichment of anthocyanins, copigments, and polymers but not a complete separation. Moreover, some widely used purification strategies (e.g., application of countercurrent chromatography) require perfluorinated reagents, which hamper the subsequent use of the so-obtained extracts in human intervention studies.[24−26] For this reason, we developed an adsorptive membrane chromatographic method to separate anthocyanins selectively from other classes of polyphenols by using a commercially available membrane system (Sartobind S, strongly acidic cation exchanger). This method does not only avoid the use of any harmful or toxic reagents, it is also easily scaled up. Application and optimization of this novel method to the fractionation of bilberry polyphenols is reported here.

MATERIALS AND METHODS

Chemicals. A standard (purity >96%) of cyanidin 3-*O*-glucoside was purchased from Extrasynthese (Genay, France). Chlorogenic acid (5-caffeoylquinic acid, 5-CQA), gallic acid, quercetin, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Fluka (Milwaukee, WI). Acetonitrile, methanol, formic acid, acetic acid (HPLC grade), and potassium persulfate (puriss.) were purchased from Sigma (St. Louis, MO).

Sample. The bilberry extract (standardized to a content of 25% total anthocyanins) was obtained from Kaden Biochemicals (Hamburg, Germany).

Adsorptive Membrane Chromatography. For the development of the membrane chromatographic method, we used two types of the strongly acidic membrane adsorbers from Sartorius Stedim Biotech (Göttingen, Germany): (a) Sartobind S 75 with a membrane area of 75 cm^2 and (b) Sartobind S IEX 150 ml with a membrane area of 0.55 m^2. These stabilized cellulose membrane adsorbers provide a net-like structure and carry sulfonic acid groups on their surfaces. They have already been used for the purification of proteins on the laboratory as well at industrial scale.[27,28] For the isolation of anthocyanins, conversion into positively charged flavylium cations by acidification is required. The flavylium cations are then retarded on the membrane adsorber surface and selectively separated from the other phenolic compounds. To protect the membrane adsorber, the solutions were prefiltered, on a small scale by using a 0.45 μm syringe filter (CHROMAFIL PET-45/25, Macherey-Nagel, Düren, Germany) and on a large scale by preinstalling a filter capsule Sartopore 2 300 with a double layer membrane, pore size of 0.45 + 0.2 μm (Sartorius, Göttingen, Germany). The sample injection (for volumes, see below) was carried out by using a 30 mL disposable syringe with a flow rate of 10 mL/min (small scale) or a peristaltic pump Tandem 1082 from Sartorius with a flow rate of 100 mL/min (large scale).

Received: November 21, 2011
Revised: February 3, 2012
Accepted: February 6, 2012
Published: February 6, 2012

dx.doi.org/10.1021/jf2047724 | *J. Agric. Food Chem.* 2012, 60, 2427−2433

Small-Scale Isolation of Anthocyanins. For separation on a small scale, the membrane adsorber type Sartobind S 75 was used. The extract was dissolved (1−10 g/L) in a freshly prepared mixture of methanol/acetic acid 19:1 (v/v) or ethanol/acetic acid 19:1 (v/v) and prefiltered as described above. Prior to the separation, the membrane adsorber had to be regenerated with 60 mL of 1 N sodium hydroxide solution. The equilibration occurred in two steps: (i) rinsing with 60 mL of a 0.01 N hydrochloric acid solution and (ii) rinsing with 30 mL of the methanolic or ethanolic extract solvent. After this, the membrane adsorber could be loaded with 80 mL (concn 1 g/L) or 8 mL (concn 10 g/L) of the extract solution. To remove the remaining copigments, the membrane adsorber was flushed with 30 mL of methanol/acetic acid 19:1 (v/v) or ethanol/acetic acid 19:1 (v/v). For elution of the anthocyanins, 80 mL of a 1 M NaCl solution mixed with methanol or ethanol in a ratio of 1:1 (v/v) was used. After elution, acidification with 1% acetic acid is required to stabilize the anthocyanins. Subsequently, the membrane adsorber could be regenerated again, and a second separation could be carried out. For storage, it was flushed with a mixture of a 1 mol aqueous NaCl solution and ethanol 2:8 (v/v) and stored at room temperature.

Large-Scale Isolation of Anthocyanins. For separations on a large scale, the membrane adsorber unit type Sartobind S IEX 150 ml was used. The bilberry extract was dissolved in concentrations of 1−10 g/L in a freshly prepared mixture of methanol/acetic acid 19:1 (v/v) or ethanol/acetic acid 19:1 (v/v) and prefiltered through a filter paper (MN 615 1/4, 270 mm, Macherey-Nagel, Düren, Germany). For protection of the membrane adsorber, a Sartopore 2 300 filter capsule was connected between the adsorber and the pumping system as described above. After regeneration and equilibration of the membrane adsorber using 2.5 L of 1 N NaOH, 2.5 L of 0.01 N HCl, and 1 L of either methanol/acetic acid 19:1 (v/v) or ethanol/acetic acid 19:1 (v/v), the membrane adsorber unit was loaded with the extract solution (for concentration, see below). Figure 1 shows the flowchart for the developed membrane chromatographic method. If it is attempted to isolate only an anthocyanin fraction, the extract solution (concn 10 g/L) can be loaded until the maximum capacity of the membrane adsorber is reached (approximately 2.6 L). In the case that also the copigment fraction should be isolated, it is recommended to add a maximum of 1 L of the extract solution. Any remaining anthocyanins in the eluate can be removed by a second membrane chromatographic step. After the membrane was rinsed with 1 L of the extract solvent, desorption of the retarded anthocyanins occurred with 1 L of a 1:1 (v/v) mixture of aqueous 1 M NaCl solution and methanol or ethanol. For stabilization of the anthocyanins, the eluate solution must be acidified with acetic acid to a final concentration of 1%.

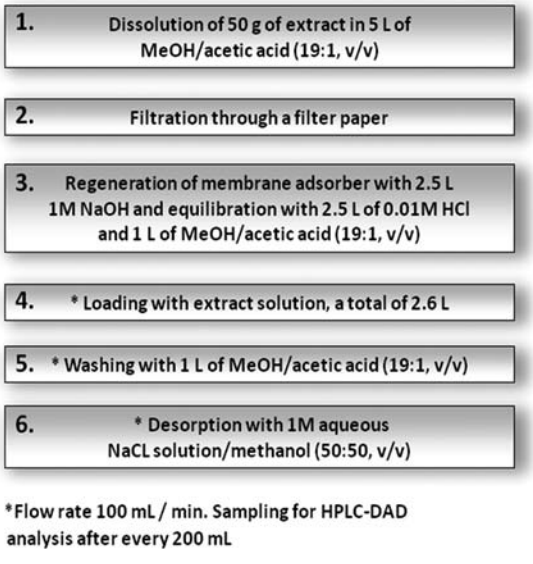


Figure 1. Flowchart for the implementation of adsorptive membrane chromatographic method for polyphenol fractionation.

Purification/Desalination of Anthocyanins and Polyphenols with Amberlite XAD-7 HP. To remove sodium chloride from the anthocyanin fractions (1−10 g), or cations from polyphenolic extracts, anthocyanins and extracts were purified using column chromatography on Amberlite XAD-7 HP (Sigma, St. Louis, MO). For this purpose, a column (120 cm × 6 cm) was filled with Amberlite XAD-7 HP, washed overnight with methanol until the washing solution was completely clear, and equilibrated with 5 L of water/acetic acid (995:5, v/v). The extracts or the sodium chloride-containing anthocyanin fractions were dissolved in 500 mL of water/acetic acid (995:5, v/v) and applied onto the column. For complete removal of salt, the column was rinsed with 3 L of water/acetic acid (995:5, v/v). Subsequently, the anthocyanin fraction was eluted with methanol/acetic acid (19:1, v/v). The solvent was removed by a rotary evaporator, and the residue was freeze-dried. For the purification of smaller fractions (0.1−1 g), the column size was 60 cm × 3 cm, and the amount of solvents was reduced accordingly.

Quantitative Analysis of Polyphenols. Prior to HPLC analysis, the samples were dissolved in water/acetonitrile/formic acid 70:20:10 (v/v/v) and filtered through a 0.45 μm syringe filter (CHROMAFIL PET-45/25, Macherey-Nagel, Düren, Germany). An aliquot of 20 μL of the sample was injected on a Luna 3 μ C-18, 250 × 4.6 mm column (Phenomenex, Aschaffenburg, Germany) with a 4 × 4 mm C-18 guard column.

The HPLC system was from Jasco (Gross-Umstadt, Germany) consisting of an Intelligent HPLC Pump PU-980 Plus, 3-Line-Degasser DG-980-50, Ternary Gradient Unit LG-980-09, Intelligent Sampler AS-950, and Multiwavelength Detector MD-151. To record and analyze the data, the system was connected to a PC system with the software ChromPass, Version 1.8.6.1 (Jasco, Gross-Umstadt, Germany).

Analyses were performed using eluent A with water/acetonitrile/formic acid 87:3:10 (v/v/v) and eluent B with water/acetonitrile/formic acid 40:50:10 (v/v/v). The gradient was set as follows: 0−20 min, 2−14% B; 20−25 min, 18% B; 9−14 min, 18−20% B; 14−30 min, 20% B; 30−42 min, 20−26% B; 42−50 min, 14% B; 25−40 min, 14−18% B; 40−45 min, 18% B; 45−70 min, 18−90% B, and then back to initial conditions during 10 min. Before the next injection, a hold time of 10 min was used. The flow rate of the mobile phase was 0.5 mL/min. Anthocyanins were detected at 520 nm and quantified using an external cyanidin 3-*O*-glucoside standard. Chlorogenic acid was detected at 320 nm and quantified using an external chlorogenic acid standard. Quercetin was detected at 350 nm and quantified using an external quercetin standard. For this, duplicate standards (between 1 and 300 μg/mL) were dissolved in solvent A (80%) and solvent B (20%), for quercetin in 100% B, to generate a six-point external standard calibration curve, with a linearity of $R^2 > 0.999$ for all.

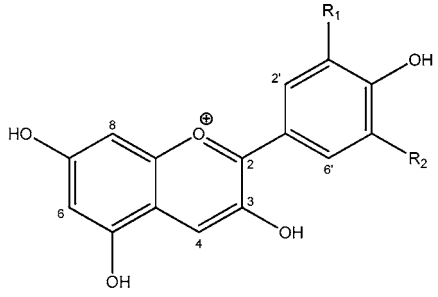
Identification of Polyphenols. For identifications, the same HPLC method as described above was applied using an Agilent HPLC system (Böblingen, Germany) equipped with a binary pump (1100 series) and an autosampler (1200 series) connected to an Esquire-LC-MS/MS ion trap mass spectrometer with electrospray ion source (Bruker Daltonics GmbH, Bremen, Germany). ESI-MS was performed in positive (anthocyanins) as well as in negative ionization mode (copigments) by using the following parameters: dry gas, N_2, 9 L/min; drying temperature, 325 °C; nebulizer, 40 psi; capillary, −3000 V; capillary exit offset, 70 V; end plate offset, −500 V; skimmer 1, 20 V; skimmer 2, 10 V; scan range, m/z 50−2200. Esquire NT 4.0 software (Bruker Daltonics) was used for analysis and data collection. Identification was based on retention time, mass, and UV/vis spectra, and comparison with commercial standards and literature.[29−32]

Analysis of Antioxidant Capacity by Using the Modified TEAC Assay.[33] The TEAC assay is based on the formation of the colored ABTS radical by reaction with potassium persulfate. Discoloration of this radical by antioxidants can be determined photometrically at 734 nm. For calibration and quantification, Trolox, a water-soluble analog of vitamin E, was used.

RESULTS AND DISCUSSION

Bilberry (*Vaccinium myrtillus* L.) is a member of the Ericaceae family. It grows in the mountains and forests of Europe as well as North America. Bilberries are a rich source of anthocyanins and other polyphenols, which are well-known as strong antioxidants.[29,30] Although numerous studies have been performed to evaluate the beneficial effects of anthocyanins, an efficient separation method for obtaining a pure anthocyanin fraction on a large scale is still missing. Previous studies have applied countercurrent chromatography,[34] but the use of harmful solvents excluded a direct use of these extracts in human intervention studies and made additional purification steps necessary. A further weak-point of the application of countercurrent chromatography (CCC) was also due to the fact that the separation is solely based on partition chromatography. This means that compounds from different polyphenolic groups with similar partition coefficients are coeluting, which hampers the preparation of a 100% pure anthocyanin fraction. In the ongoing discussion on the biological effects of the different classes of polyphenols, it is essential to ensure the purity of the testing material. In view of this, we have attempted to develop a novel strategy, which allows the fractionation of bilberry polyphenols in pure fractions of anthocyanins, copigments, and polymeric compounds.

Analysis of Anthocyanins and Copigments. In a first step, we characterized 15 different anthocyanins in the bilberry extract, that is, 3-*O*-glucosides, 3-*O*-galactosides, and 3-*O*-arabinosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin (for structures, see Figure 2) by using HPLC-DAD-ESI-MS.[29−32] The quantification of anthocyanins with HPLC-DAD was performed as cyanidin 3-glucoside equivalents by using commercially available cyanidin 3-glucoside as a reference standard at a wavelength of 520 nm. The total content of the 15 main anthocyanins of the extract was 27.5%. Figure 3 shows the anthocyanin composition of the bilberry extract. In addition to the identified 15 anthocyanins, a number of other copigments like phenolic acids and flavonols were detected by HPLC-DAD-ESI-MS analysis (cf., Table 1).



	R_1	R_2	MW [g/mol]
Cyanidin (Cy)	OH	H	287
Delphinidin (Del)	OH	OH	303
Peonidin (Peo)	OCH_3	H	301
Petunidin (Pet)	OH	OCH_3	317
Malvidin (Mal)	OCH_3	OCH_3	331

Figure 2. Structures of bilberry anthocyanidins.

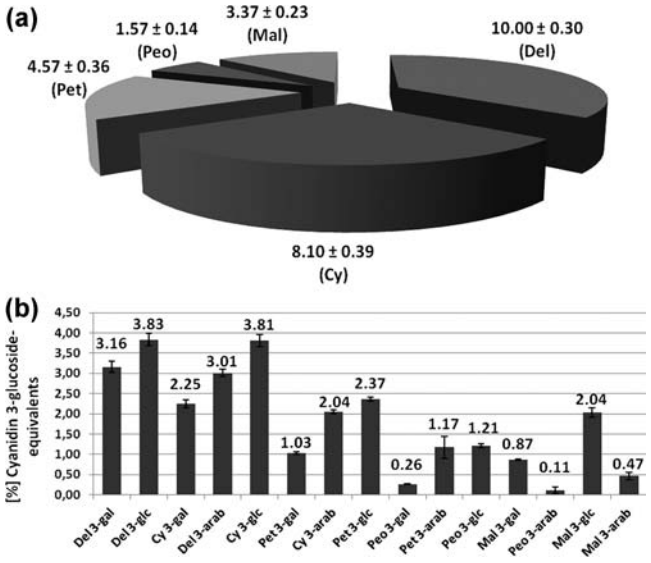


Figure 3. Distribution of anthocyanin glycosides according to their aglycones (a) and anthocyanin profile (b) in bilberry extract [%]. For abbreviations, see Figure 2: gal, galactoside; glc, glucoside; arab, arabinoside.

Isolation of Anthocyanins Using Adsorptive Membrane Chromatography. To isolate and separate the anthocyanins from the bilberry extract, the following membrane chromatographic method was developed. For preliminary experiments, the membrane adsorber Sartobind S 75 was used. During the loading, washing, and elution step, every 10 mL, a sample of 1 mL of the solution was collected, dried under nitrogen (to prevent oxidation of polyphenols), and analyzed by HPLC-DAD. It could be shown that the positively charged flavylium cations were efficiently adsorbed by the negatively charged sulfonic acid groups on the membrane surface during the loading, while all uncharged molecules pass the net-like membrane structure. When using an extract concentration of 1 g/L and a volume of <80 mL, more than 99.5% of the anthocyanins was adsorbed. Thereafter, the maximum capacity of the membrane adsorber was reached, and the anthocyanin retention decreased (Figure 4). With regard to copigments, the concentration of retained chlorogenic acid and quercetin was quantitatively determined in the membrane adsorber leaving extract solution. It was found that retention of chlorogenic acid was <10% and quercetin <25%, in both cases, if using methanol/acetic acid 19:1 (v/v) or ethanol/acetic acid 19:1 (v/v) extract solution. It is noteworthy that membrane chromatography using aqueous extract solutions or juices after acidification with HCl to pH 2 is also possible, but the retention of lipophilic compounds is considerably higher (>50%). Therefore, the use of methanolic or alternatively ethanolic extract solutions (for food supplements) for the described membrane chromatographic process is advantageous. To remove the small amount of adsorbed copigments, the membrane adsorber can be rinsed with a mixture of methanol/acetic acid 19:1 (v/v) or ethanol/acetic acid 19:1 (v/v). After a volume of 30 mL, no further copigments can be detected in the washing solution.

For the subsequent desorption of the retarded anthocyanins, an aqueous solution of 0.1 M NaCl mixed with methanol or

dx.doi.org/10.1021/jf2047724 | J. Agric. Food Chem. 2012, 60, 2427−2433

Table 1. HPLC-DAD-ESI-MS Data and Properties of the Detected Copigments in Bilberry Extract

	compounds	λ_{max} [nm]	ESI-MS (negative mode) $[M-H]^-$	fragments $[m/z]$	t_R [min]
1	gallic acid	271	169	125	8.03
2	gallocatechin	271	305	261, 219, 179, 165, 139, 137	8.39
3	protocatechuic acid	291, 259	153	179, 135	13.2
4	catechine	279	289	245, 179, 125	14.05
5	epigallocatechine	271	305	261, 219, 179, 165, 139, 137	14.87
6	5-hydroxy-vanillic acid	275	183	168, 139, 124	17.23
7	depside	279	335	183, 169, 165, 139	20.2
8	caffeic acid derivative	315, 295	341	179, 135	21.12
9	chlorogenic acid (5-CQA)	299, 327	353	191	22.97
10	proanthocyanidin dimer	279	577	559, 451, 425, 407, 289	24.4
11	caffeic acid	323, 295	179	135	26.39
12	epicatechine	279	289	245, 179, 125	28.48
13	phloroglucinol aldehyde	287	153		29.41
14	caffeoylshikimic acid	327, 295	335	179, 161, 135	32.3
15	coumaroylquinic acid (5-pCouQA)	311, 291	337	191, 163	32.39
16	depside	295, 267	319	183, 165, 153, 139	35.35
17	*p*-coumaric acid	311, 247	163	119	40.3
18	myricetin-galactoside	351, 295	479	316	40.44
19	myricetin-glucoside	351, 295	479	316	42.33
20	feruloylquinic acid (5-FQA)	323, 295	367	193. 191, 173	51.4
21	quercetin-galactoside	351, 259	463	301	55.91
22	quercetin-glucoside	351, 259	463	301	56.71
23	quercetin-glucuronide	351, 259	477	301	57.45
24	coumaroyl-iridoid isomer 1	311, 295	535	491, 371, 329, 311, 191, 163	57.71
25	coumaroyl-iridoid isomer 1	311, 295	535	491, 371, 329, 311, 191, 163	58.52
26	quercetin-arabinoside	351, 295	433	301	61.52
27	quercetin-rhamnoside	347, 295	447	301	62.85
28	myricetin	375, 295	317	179, 151, 137, 107	63.6
29	coumaric acid derivative	311, 291	411	(249), 163, 145, 119	68.11
30	quercetin	371, 295	301	179, 151, 107	70.91

ethanol in a ratio of 1:1 (v/v) was applied. Pure aqueous NaCl solutions show a much lower eluting power. By using a volume of 80 mL, the anthocyanins could be completely desorbed. Higher salt concentrations (e.g., a 1 M alcoholic NaCl solution) accelerate this process and give a complete elution of the anthocyanins already after 8 mL. The so-obtained anthocyanin fraction, which was immediately stabilized with 1% acetic acid

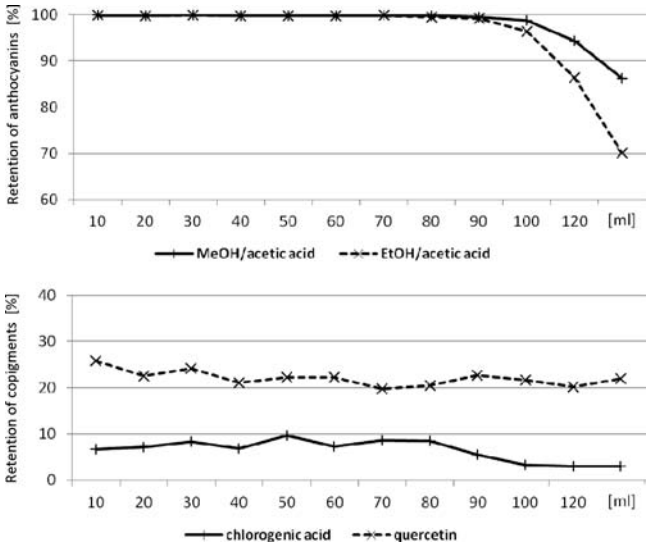


Figure 4. Retention of anthocyanins and copigments during the membrane chromatographic separation (small scale), depending on extract volume, determined by comparison with the initial concentration of the extract solution. Extract dissolved in methanol/acetic acid 19:1 (v/v) and ethanol/acetic acid 19:1 (v/v), concentration [1 g/L].

solution, contained 99.7% of the initial anthocyanin concentration and was free of copigments (Figure 5). After

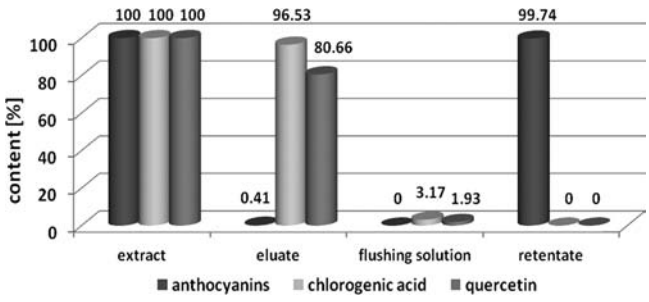


Figure 5. Content (in % of original concentration in extract solution) of anthocyanins and copigments in the fractions obtained after membrane chromatography.

concentration in vacuo and lyophilization, desalination of the anthocyanin fraction was achieved with Amberlite XAD-7 HP as described above in the Materials and Methods. After removal of the salt, 25 mg of the copigment free fraction of anthocyanins was isolated from 80 mg of the bilberry extract.

Scale-Up of the Developed Method. To isolate the anthocyanin fraction on a preparative scale, a membrane adsorber unit type Sartobind S IEX 150 ml was used. Again, the anthocyanin content was monitored by HPLC-DAD analyses during the loading, washing, and elution steps. Until a volume of 800 mL of extract solution (concentration 10 g/L), over 98% of the bilberry anthocyanins was adsorbed, because less than 2% of the original concentration of anthocyanins was found by HPLC-DAD analysis in the eluate. Afterward, the retention of anthocyanins decreased, and the absorber reached its maximum capacity at 2600 mL. At this point, the concentration of anthocyanins in the eluate corresponded to the initial concentration in the extract (Figure 6).

For anthocyanin isolation, the sample can be added until the maximum capacity of the membrane adsorber is reached at

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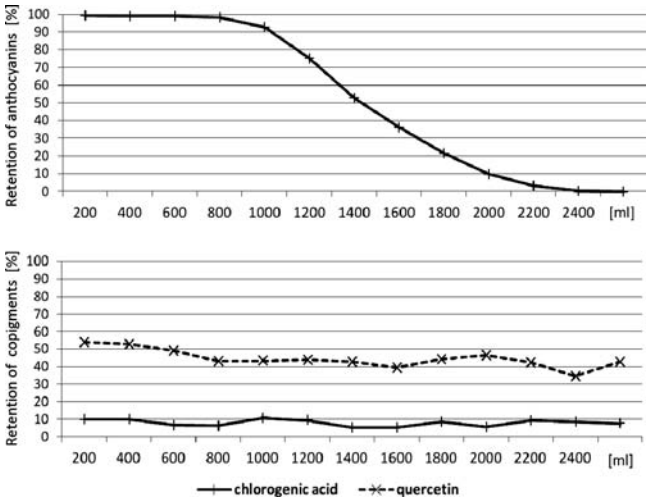

Figure 6. Retention of anthocyanins and copigments during the membrane chromatographic separation (large scale), depending on extract volume, determined by comparison with the initial concentration of the extract solution. Extract dissolved in methanol/acetic acid 19:1 (v/v) and ethanol/acetic acid 19:1 (v/v), concentration [10 g/L].

2600 mL; however, if also the copigment fraction is required, it is recommended to add a maximum of 1 L of extract solution (corresponding to 10 g of dry extract). If necessary, small amounts of anthocyanins in the copigment fraction are easily removed by a second membrane chromatography. This allows adsorption of over 99.8% of the original anthocyanin content from the extract. It is also possible for this purpose to switch two or several membrane adsorbers in series. During the entire membrane chromatography, the content of chlorogenic acid in the eluate was between 90% and 95% of the initial extract concentration, and the concentration of quercetin was between 50% and 65%. This means that 5−10% of chlorogenic acid and 35−50% of quercetin initially retained by the membrane adsorber unit (Figure 6) need to be removed by a rinsing step. Approximately 1 L of a mixture of methanol/acetic acid 19:1 (v/v) or ethanol/acetic acid 19:1 (v/v) was sufficient for a complete copigment elution from the adsorber unit. For the subsequent anthocyanin desorption, 1 L of an aqueous 1 M NaCl solution mixed with methanol or ethanol in a ratio of 1:1 (v/v) was required, with the majority of the anthocyanins already eluting in the first 600 mL.

Repetition of the described membrane chromatographic process or connection of two membrane adsorbers in series allowed an isolation of 3.71 g of a pure anthocyanin fraction from 10 g of the bilberry extract. Concentration and desalination were performed as described for the small-scale experiment.

If the sample load was higher than the maximum capacity of the membrane adsorber, 4.1 g of the anthocyanin fraction was obtained. The membrane (with an adsorption surface of 0.55 m^2) had a binding capacity of 7.4 g/m^2 for bilberry anthocyanins. Even after 30 separations, the results were reproducible, and there was no decrease in the absorption capacity of the adsorber.

Figure 7 shows the HPLC chromatograms of the original bilberry extract (1) and the anthocyanin (2) and the eluate fraction (3) after separation by membrane chromatography, respectively. On the left side are depicted HPLC-DAD measurements of these fractions at 280 nm (a), and on the right side are shown measurements at 520 nm (b). Because anthocyanins have two absorption maxima at 280 and 520 nm, they can be distinguished very well from the copigments by using this comparison, because the copigments only absorb at

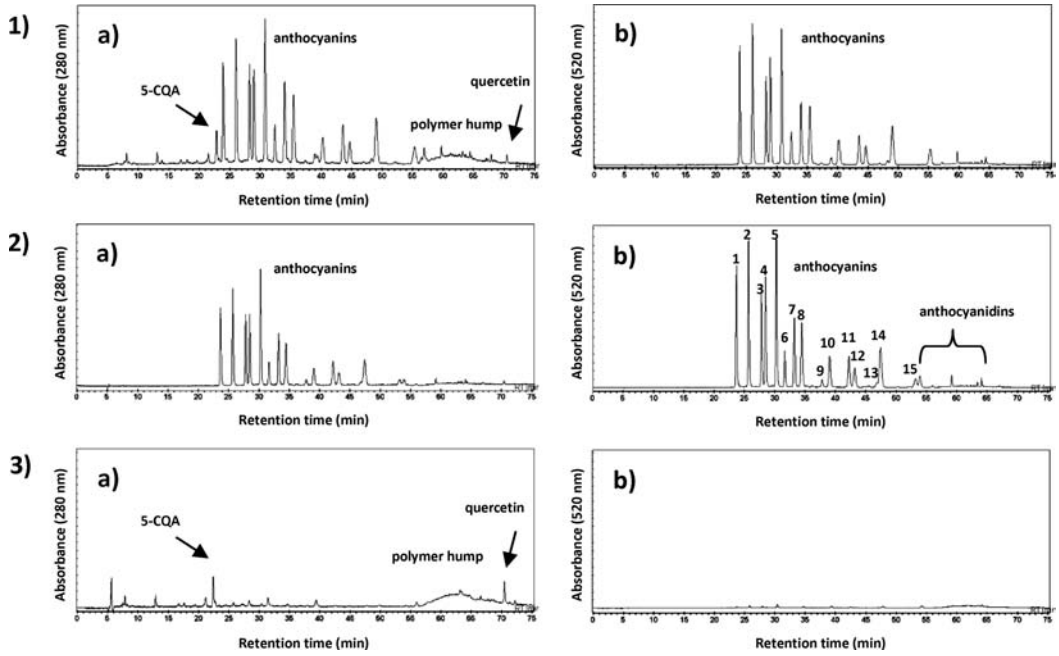

Figure 7. Chromatograms of the original bilberry extract (1) and of the anthocyanin (2) and eluate fraction (3) after separation by membrane chromatography. Left, HPLC-DAD measurements at 280 nm (a); and right, at 520 nm (b). Delphinidin 3-galactoside (1), delphinidin 3-glucoside (2), cyanidin 3-galactoside (3), delphinidin 3-arabinoside (4), cyanidin 3-glucoside (5), petunidin 3-galactoside (6), cyanidin 3-arabinoside (7), petunidin 3-glucoside (8), peonidin 3-galactoside (9), petunidin 3-arabinoside (10), peonidin 3-glucoside (11), malvidin 3-galactoside (12), peonidin 3-arabinoside (13), malvidin 3-glucoside (14), and malvidin 3-arabinoside (15).

dx.doi.org/10.1021/jf2047724 | J. Agric. Food Chem. 2012, 60, 2427−2433

280 nm. Thus, the chromatogram (1a, 280 nm) of the extract shows not only the peaks of anthocyanins and copigments, but also the so-called "polymer hump" from 55 to 70 min caused by the polymeric compounds present in the extract. On the contrary, the chromatogram of the extract at 520 nm (1b) shows only the absorbance of the anthocyanins at 520 nm. The almost identical chromatograms 2a and 2b of the anthocyanin fraction show only anthocyanins, whereas copigments and polymers are not detectable in this fraction. It is also obvious that the anthocyanin profile has not changed. This demonstrates that all of the 15 bilberry anthocyanins can be recovered completely after this novel separation step employing a membrane adsorber unit. The chromatogram of the eluate fraction at 520 nm (3b) shows that anthocyanins are not present in this fraction. Only copigments and polymers could be detected at 280 nm (3a). These chromatograms demonstrate the complete separation by membrane chromatography of anthocyanins and copigments from the bilberry extract.

After the separation of anthocyanins from the extract, copigments were obtained from the eluate fraction by separation of the polymeric constituents according to the following flowchart (Figure 8). For this purpose, the eluate

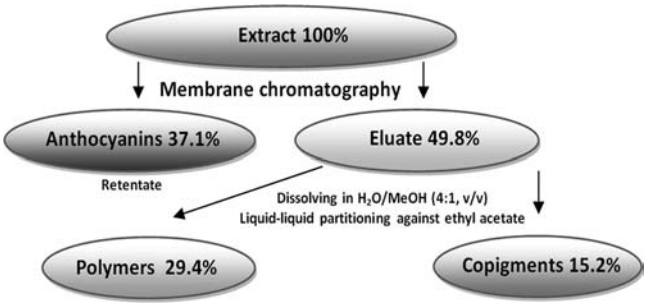

Figure 8. Flowchart to isolate highly purified anthocyanin, polymer, and copigment fractions from the bilberry extract.

fraction was dissolved in 1 L of a water/methanol mixture 4:1 (v/v). Repetitive liquid–liquid extraction (four times) with 1 L of ethyl acetate allowed separation of less polar copigments from the polar residue consisting of polymers.

Determination of the antioxidant capacity of the so-obtained three fractions, that is, anthocyanins (37% of total extract), copigments (15%), and polymers (29%), was performed by using the modified TEAC-assay.[33] The anthocyanin fraction showed the highest antioxidant capacity (4.6 mmol Trolox/g), followed by the copigment fraction (3.2 mmol Trolox/g), and the polymeric fraction (2.1 mmol Trolox/g), respectively.

This novel adsorptive membrane chromatographic method is also suitable for the isolation of anthocyanins from other fruits and has already been successfully applied to the fractionation of polyphenols from cranberry, blackberry, and pomegranate. The scale-up to industrial scale is in progress.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This research project was supported by the German Ministry of Economics and Technology (via AiF) and the FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn), project AiF 15610 N.

We thank Mr. W. Demmer and Mr. M. Wörner from Sartorius Stedim Biotech (Göttingen, Germany) for helpful discussions.

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